PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT	:	ROTHSCHILD et al.
SERIAL NO	:	09/950,022
FILED	:	September 10, 2001
TITLE	:	NOVEL PRKAG3 ALLELES AND USE OF THE SAME AS GENETIC MARKERS FOR REPRODUCTIVE AND MEAT QUALITY TRAITS
Grp./A.U.	:	1634
Examiner	:	SWITZER, Juliet Caroline
Conf. No.	:	1703
Docket No.	:	P04668US03

DECLARATION OF DR. MAX F. ROTHSCHILD UNDER 37 CFR §1.131

Mail Stop Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Max F. Rothschild, declare and say:

A. Purpose of Declaration

1. That I am a named inventor for the above-identified application.

2. That this declaration is to establish conception of the invention in this application in the United States prior to May 19, 2000, the effective publication date of the prior art reference Milan et al. *Science*, May 19, 2000, Vol. 288, pages 1248-1251, that was cited by the Examiner in the Office Action of May 15, 2003.

B. Facts and Documentary Evidence

3. That to establish this conception, the following attached documents are submitted as evidence:

(a) Exhibit A: Reproduction of a research report for NPPC and Industry Consortium Members (7 pages).

(b) Exhibit B: Table 3 shows markers chosen and genotyped (2 pages).

(d) Exhibit C: Table 4 shows estimated significance level found on Chromosome 15 for QTL for various growth and meat quality traits (2 pages).

(e) Exhibit D: Graphs of chromosomes 15 showing QTL for meat quality traits (4 pages).

4. That from these documents, all of which were in existence prior to May 19, 2000, it can be seen that the invention in this application was at least conceived prior to the date of May 19, 2000, a date earlier than the effective publication date of the reference. Dates on the documents have been redacted.

5. That specifically, the documents of Exhibits A-D establish conception and reduction to practice of the invention as described and claimed in the application:

(a) Exhibit A shows that prior to May 19, 2000, the effective publication date of the prior art reference Milan et al., a 3-generation source family was developed using 2 Berkshire grand sires and 9 Yorkshire grand dams to detect quantitative trait loci (QTL) for meat quality traits and glycolytic potential in pigs, and that a total of 525 F2 progeny from 65 matings from 65 F1 litters were produced.

(b) Exhibit A shows that prior to May 19, 2000, the effective publication date of the prior art reference Milan et al., animals were genotyped for 125 microsatellite markers covering the genome, beginning in early 1999. Table 3, Exhibit B, shows the markers chosen and genotyped.

(c) Exhibit C (Table 4) shows that prior to May 19, 2000, the effective publication date of the prior art reference Milan et al., Applicant identified QTLs significant at the 5% chromosome wise level.

(d) That prior to May 19, 2000, the effective publication date of the prior art reference Milan et al., on chromosome 15, a total of 11 QTL for meat quality traits were seen which exceeded the 5% chromosome wise significance level. Of these, glycogen was one of three that exceeded the 5% genome wise significance level. Berkshire allele resulted in lower glycogen. Each of the QTL accounted for from 2.5% to 5.6% of the variance.

(e) Exhibit D shows that at a time prior to May 19, 2000, the effective publication date of the prior art reference Milan et al., Applicant identified that a significant QTL effect existed for meat quality traits and glycolytic potential in F2 animals derived from a cross (Berkshire x Yorkshire) of two breeds known not to contain the RN^- mutation unlike that disclosed in Milan et al. These included QTL for average glycogen content in the muscle and in general the meat quality characteristics of pigs which include ultimate pH and color.

C. Diligence

6. That from the time of this conception, a time just prior to the effective publication date of the reference to the filing of Applicant's application as identified in the caption of this declaration, Applicants diligently moved towards a method of assaying for the presence of a marker correlated to average glycogen content in the muscle and in general the meat quality characteristics of pigs.

7. That Exhibit E (10 pages) (the dates have been redacted) is reproductions of notebook entries of PCR-RFLP tests entered in late May 2000 and entered up to the filing of the application. The test was developed to find the causative mutation of the phenotypic variation of glycogen, lactate, and glycolytic potential and in general the meat quality characteristics in pigs.

8. That specifically, Exhibit E shows:

(a) In late May 2000, a PCR-RFLP test for the PRKAG3-199 mutation that included using primers that flanked the mutation, digesting the PCR product with *BshHI* and separating them on a gel;

(b) In early July 2000, a PCR-RFLP test for the PRKAG3-52 mutation including digestion with the *HPhI* for the mutation found at position 154 (codon 52) of the PRKAG3 gene;

(c) In early July 2000, a test of the Berkshire x Yorkshire (B x Y) F2 animals for the RN-*HphI* marker;

(d) In mid July 2000, correlation of PRKAG3 mutation at codon 52 with lactate;

(e) In mid July 2000, digestion with *BsaHI* to differentiae the individual;

(f) In mid July 2000, correlation of the PRKAG3 mutation at codon 199 with glycolytic potential.

9. That an assay using a PCR-RFLP test was reduced to practice in July 2000 for each mutation tested in the F2 progeny for glycogen, lactate, and glycolytic potential.

10. That work continued on the RFLP tests up to a time shortly before filing of the above-identified application, including digestion with the RN-*HPhI* for the mutation found at position 154 (codon 52) of the PRKAG3 gene.

11. That in early August 2000, Applicants executed an Intellectual Property Disclosure & Record statement to the assignee (Exhibit F-13 pages). The dates have been redacted. Exhibit F shows the inventors, title of the invention/creation, a brief description of the invention, commercial uses, and prior art, and a preliminary write up of results.

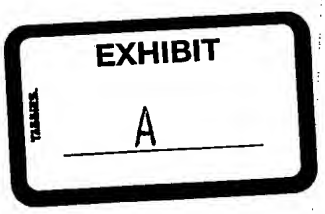
12. That the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be

the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Sept 15, 2007
Date

Max F. Rothschild, Ph.D.

A Molecular Genome Scan Analysis to Identify Genes Influencing Muscle Quality in the Pig



Summary:

Genome scans can be employed to identify chromosomal regions and eventually genes (quantitative trait loci or QTL) that control quantitative traits of economic importance. A three-generation resource family was developed using two Berkshire grand sires and nine Yorkshire grand dams to detect QTL for meat quality traits in pigs. A total of 525 F2 progeny from 65 matings from 65 F1 litters were produced. All F2 animals were phenotyped for birth weight, 16 day weight, growth rate, backfat, loin eye area, drip loss, water holding capacity, firmness, color, marbling, percent cholesterol, ultimate pH, fiber type and several sensory panel and cooking traits. Animals were genotyped for 125 microsatellite markers covering the genome. Linkage analysis was performed using CRIMAP version 2.4 (Green et al. 1990). Regression interval mapping (Haley et al. 1994) was used for QTL detection. Significance thresholds were determined by permutation tests. Significant QTL at the chromosome wise 5% level were detected for a total of over 100 growth (chromosomes 1, 2, 3, 4, 6, 7, 8, 9, 11, 13, 14, X), backfat (chromosomes 1, 4, 5, 6, 7, 10, 13, 14) and meat quality traits (chromosomes 1, 2, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 17, 18, X). Additional marker analysis and examination for positional candidate genes is underway. This research was supported by an industry consortium consisting of National Pork Producers Council, Iowa Pork Producers Association, Iowa Purebred Swine Council, Babcock Swine, Danbred USA, DEKALB Swine Breeders, PIC, Seghersgenetics USA, and Shamrock Breeders.

Introducti n:

The techniques of molecular biology and molecular genetics have rapidly progressed. These methods, coupled with advances in human genetics, have opened new vistas for investigators wishing to identify genes that control quantitative traits (quantitative trait loci or QTL). Also, over the past several years, a great deal of progress has been made in development of genetic maps in the pig. Already, a large international mapping effort (Archibald et al., 1994), a USDA/ARS effort (Rohrer et al., 1996) and a U.S. coordinated effort (Rothschild, 1994) have produced several genetic linkage maps for the pig. In total, approximately 1,900 genes (as of January 2000) have now been mapped in the pig, with a majority of these being anonymous molecular markers called microsatellites. These efforts to place genes and markers on chromosomes are already paying dividends in the search for molecular genetic markers for traits such as growth and backfat (Andersson et al., 1994; Rothschild et al., 1995), meat quality (Milan, et al., 1995) and reproduction (Rothschild et al., 1996). Several recent QTL studies have reported QTL for some growth and meat quality studies in crosses involving exotic breeds (Andersson-Eklund et al., 1998; Moser et al., 1999; Paszek et al., 1999; Rohrer and Keele, 1998; Rothschild et al., 1995; Wang et al., 1998).

Results from the NPPC Genetic Evaluation Program (Goodwin, 1995) revealed that considerable differences in meat quality exist between breeds and that the Berkshire breed, in particular, has very positive meat quality traits. The general use of genes and genetic markers makes it possible to localize the QTL responsible for meat quality traits. However, useful resource families using commercial lines did not exist at the initiation of this project. It was determined that a three generation resource family needed to be developed with the Berkshire breed in order to determine the chromosomal regions and genes responsible for differences in meat quality traits. Such a family would provide enormous opportunities to fully extend the previous research to pinpoint the location of genetic markers associated with meat quality in the pig. The research presented here therefore lays the foundation for the study of the genetics of meat quality.

Objectives:

The overall goal of this research was to identify specific chromosomal regions associated with meat quality.

Specific Objectives

1. Collect muscle quality data on approximately 525 F2 animals from the Berkshire x Yorkshire muscle quality resource family.

2. Perform a total genome scan using 90 anonymous genetic markers (approximately 4 per chromosome). This was later amended to add 25-35 markers so that the total is now 125 markers.

3. To perform statistical QTL analyses to determine chromosomal regions associated with muscle quality traits.

4. Finalize analyses and report results to NPPC. Work with producer groups and the breeding and packing industries to transfer useful results.

Procedures to Complete Objectives:

Family structure:

A total of 2 Berkshire boars (chosen with NPPC guidance) and 9 Yorkshire females were used to produce 9 useful F1 litters. Semen from boar studs was used and sows mated at the ISU Swine Breeding Farm. The two boars used were Casino and Count. From the F1 litters, 8 boars and 28 females were chosen to produce 65 litters of 525 F2 animals for genetic and meat trait analysis.

Traits measured:

Performance data collected included birth weight, 16d weight, ADG from birth to 16 days of age and ADG from weaning to slaughter. Pigs were weighed at weekly intervals and sent to market at approximately 240 lbs. After slaughter, carcass traits were evaluated according to National Pork Producers Council procedures (NPPC, 1991). These data included carcass weight, visual scores for loin muscle marbling, color and firmness in the plant cooler and in Ames, ultimate pH, Minolta reflectance and Hunter L. color scores for ham and loin. Water holding capacity was measured using a piece of filter paper (higher weight is less water holding capacity) and drip loss was calculated using two separate cubes of meat and by collecting the drip over 72 hrs. In addition, a loin chop was taken from each carcass and samples from it were used to evaluate lipid content. Also measured was Star Probe tenderness and sensory taste panel evaluations for tenderness, chewiness, juiciness, flavor and off flavor of the cooked loin were collected. See Table 1 for a description of the traits.

DNA isolation and genotyping:

Blood samples were collected from all F2 animals and parents and grandparents and DNA samples collected. Likely parentage (or collection) problems existed on less than 20 F2 animals and these were discarded for analyses. We believe this number is extremely low (about 4%).

We sub-contracted the genotyping to a commercial laboratory (GeneSeek Inc, Lincoln NE) to speed the process and minimize costs. We did this after receiving bids for this work in . This had worked well for another NPPC funded project. We tested about 180 markers on the F0 and F1 animals and ended up with the final 125 markers to use in the project for genotyping the F2 animals, which began in . See Table 3 for markers chosen and genotyped.

QTL analyses:

Linkage analyses were computed using CriMap. The maps were then used for the QTL analyses. The QTL were identified for the 18 autosomes and the X chromosome using the least squared regression interval mapping program developed by Haley and Knott (1994). The models used included sex and year-season and the covariable live weight for carcass traits and the covariable litter size for birth and 16 day weight and for ADG from birth to 16 days. For meat quality and sensory traits the effect of year-season was removed and the effect for slaughter date was added

Significance levels were calculated using the permutation test developed by Churchill and Doerge (1994). This was computed for both the individual chromosome and the genome wise level based on 10,000 random permutations of the data. Individual chromosome significance levels (P<.05) ranged from 4.34 to 5.32, while genome-wise significance thresholds were 8.22 (P<.05) and 9.96 (P<.01). The method of Lander and Kruglyak (1995) was also used for comparison sake and resulted in genome-wise thresholds of 10.4 (P<.05) and 12.30 (P<.01).

Results:

General meat quality results

Results from the samples conformed to the usual range of measurement scores. The arithmetic means and correlations among the traits can be found in Tables 1 and 2. Considerably more effort to understand the relationships between the traits is underway. A full publication is planned by the team to cover this area of work.

Chromosome map results

Marker mapping results are presented by chromosome in Table 3. Total map length was 20.8 Morgans and compares well to previous maps. In all cases but one the map order was the same as that of the USDA map (Rohrer et al., 1996). The exception was a switch in order for chromosome 2 between SW2157 and SW1408. In our map these markers are 4 cM apart while the order is reversed in the USDA map and they are 2 cM apart. While the original plan for the map was to have 90 markers and average distances of 30-40 cM, funds allowed for more markers to be added. Average maker distances were 17 cM but a total of 8 gaps existed of greater than 30 cM, despite efforts to include more markers. Finding markers for these gaps was limited by choosing ones that were easy to use and informative. More effort could be devoted to this in the next stage. Information content of the markers was calculated on an individual method basis (IIC) and on a linked marker basis (EIC) and this information is presented in Table 3. Information content on a linked marker basis includes information from flanking markers, in addition to information from the marker itself, for determining the breed origin of alleles at the marker for individual F2 progeny. This is what was used in the QTL mapping program.

QTL results

QTL results for those significant at the 5% chromosome wise level are in Table 4 and in the figures that are in a separate file. On occasion extra graphs are shown to show some supporting evidence that is close to meeting the significance level. They are presented on a per chromosome basis. The genome-wise significance thresholds were 8.22 (P<.05), 9.96 (P<.01) and 12.50 (P<.001).

Chromosome 1

A total of 10 QTL (primarily for fat traits) were observed that exceeded the 5% chromosome-wise significance level, with one of these (marbling) exceeding the 5% genome-wise significance level and two (loin eye area, tenth rib BF) exceeding the 1% genome-wise significance level. They primarily centered from 29 to 66 cM on our map. An exception was drip loss at 90 cM and a secondary QTL for loin eye area at 105 cM. Berkshire alleles were generally associated with less fat, a larger loin eye area and less drip loss when compared to Yorkshire alleles for the QTL

Chromosome 15

A total of 11 QTL for meat and sensory quality traits were seen on chromosome 15 which exceeded the 5% chromosome wise significance level. Of these, three (Hormel ham pH, lab loin pH and average glycogen) exceeded the 5% genome wise significance level and one (Hormel loin pH) was significant at the 1% genome wise level. Berkshires alleles resulted in better reflectance scores, higher pH, lower glycogen, higher tenderness score, less star probe force and more intense flavor for QTL in the area of 44-96 cM. Each of these QTL accounted for from 2.5 to 5.6% of the variance.

Discussion:

Map lengths and marker order conformed well to previous results. In some instances there was difficulty in filling gaps and a few large gaps remain. No additional markers were chosen at the end of phase one as funds were exhausted and time was too short to add more markers. Furthermore, since the original 90 markers used were advanced by an additional 35 markers it is clear the original objectives had been met. With additional funds some new markers could be tested to fill these gaps.

QTL effects existed for nearly all traits. They varied in size though most accounted for 3-5 % of the total variance. Some QTL exceeded this considerably and reached 10%. Both breeds had favorable QTL on separate chromosomes for quality traits. In addition there was some evidence on several chromosomes that cryptic alleles existed which favored the breed least expected to have them. If several of these could be used in marker assisted selection then the improvement could be considerable. These results will allow others to attempt to identify the individual genes responsible for the traits. One final comment is that we did observe some overdominance. This could represent real overdominance or be due to the QTL effects observed here being due to two or more tightly linked QTL. This can be more accurately assessed once the genes responsible are identified.

Table 3. Markers and their approximate map position in this QTL map in cM relative to positi n of the first marker

MARKER	Chrom s me	Position	Number of alleles	IIC*	EIC*

MARKER	Chr	m	s	me	Position	Number of alleles	IIC*	EIC*
SW1416	15				0	5	0.97	0.98
S0148	15				21.5	5	0.89	0.93
SW964	15				38.2	5	0.86	0.92
SW1683	15				59.3	4	0.70	0.88
SW936	15				69.1	4	0.76	0.91
SW1983	15				80.5	7	0.90	0.94
SW1119	15				96	5	0.61	0.83

For comparison see USDA Map (Rohrer et al., 1996).

*IIC: Individual information content based on this marker only.

*EIC: Effective information content including information on linked markers.

Table 4. Evidence for QTL for various growth and meat quality traits by chromosome. Estimated significance level (F value) for trait QTL

SSC	Trait	F-value	Location	Additiv		Dominance		% QTL	

SSC	Trait	F-value	Location	Additiv effe t	S.E.	D minance effect	S.E.	% QTL var
15	Hormel Loin Hunter	6.31	96	-1.065	0.321	0.624	0.500	3.16
15	Lab Loin Hunter	5.04	66	-0.677	0.216	0.166	0.327	2.46
15	Lab Loin Minolta	6.30	66	-0.727	0.207	0.165	0.313	3.05
15	Hormel Ham pH	8.42	72	0.054	0.014	-0.021	0.021	4.00
15	Hormel Loin pH	12.15	76	0.053	0.011	-0.005	0.015	5.61
15	Lab Loin pH	9.05	45	0.043	0.012	-0.038	0.019	5.14
15	Average Glycogen (umol/g)	8.25	65	-0.771	0.222	0.708	0.337	4.27
15	Average Glycolytic Potential (umol/g	6.21	67	-3.666	1.048	0.766	1.587	2.95
15	Tenderness Score	5.22	44	0.240	0.084	-0.204	0.135	3.00
15	Average Star Probe Force (kg)	5.25	42	-0.166	0.054	0.092	0.085	2.88
15	Flavor score	6.41	91	0.355	0.114	-0.336	0.183	3.73

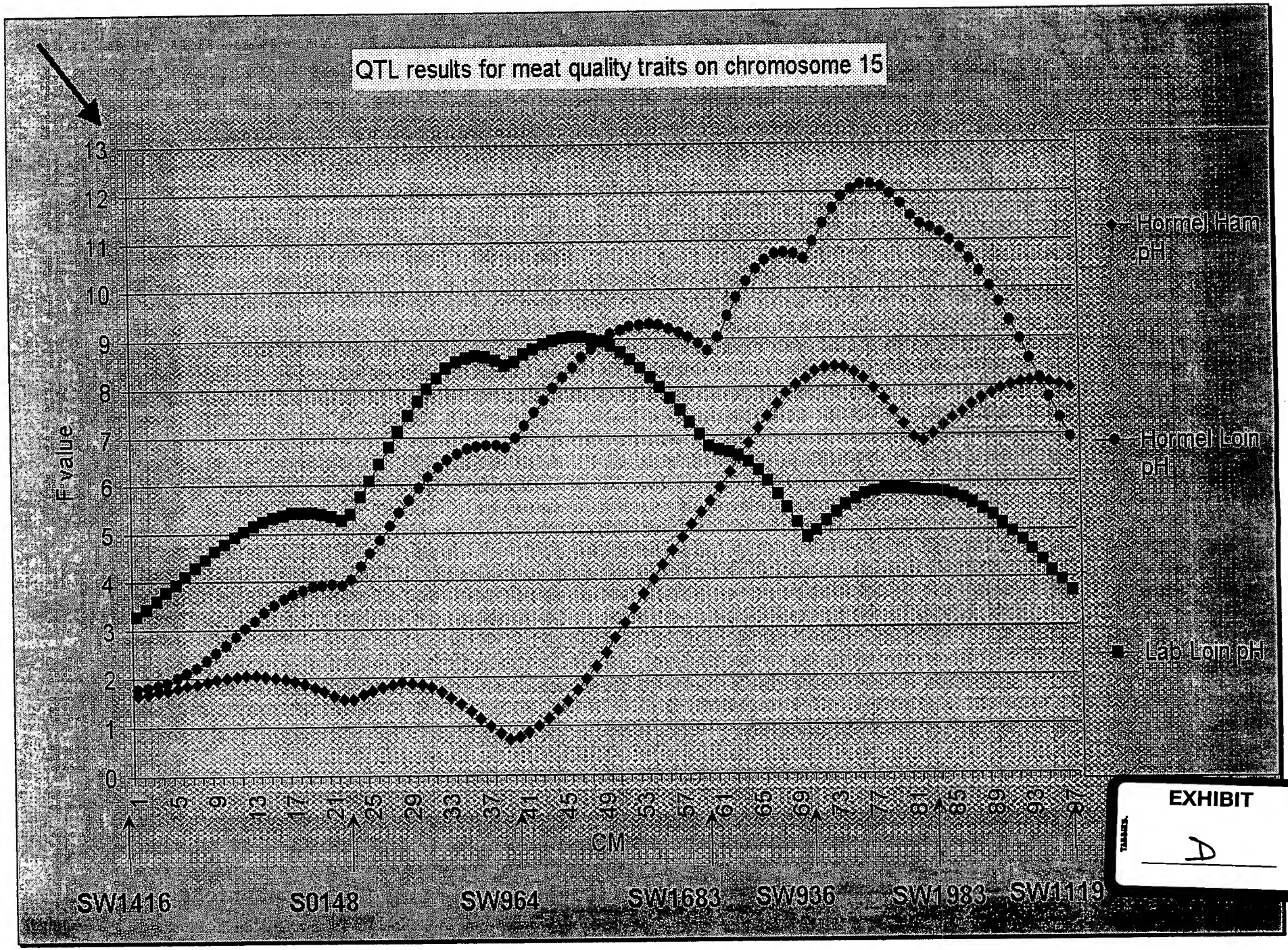
Chromosome-wise F-statistic thresholds at the 5% level, as determined by permutation test were as follow:

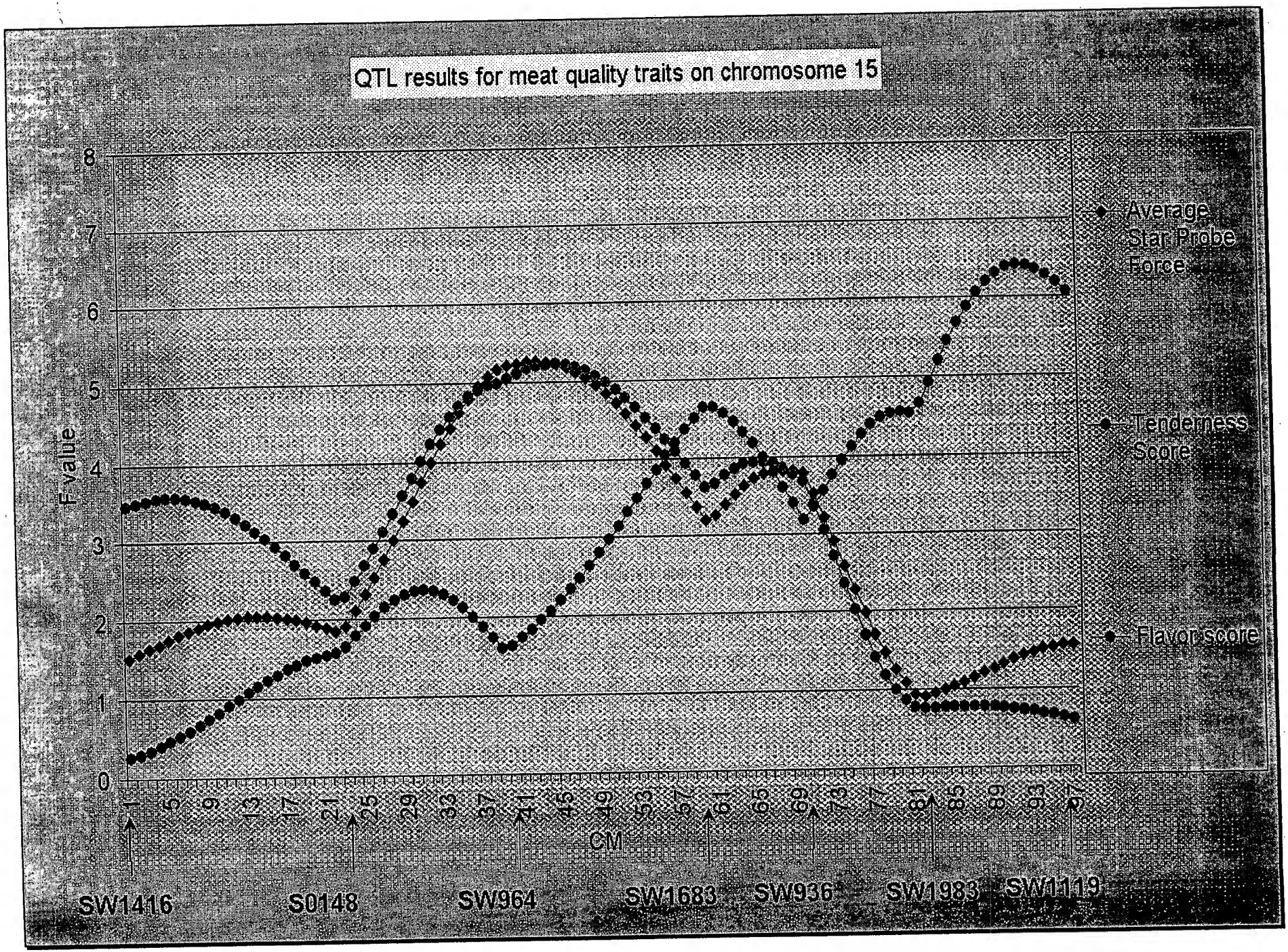
(1) 5.08, (2) 5.12, (3) 5.14, (4) 5.14, (5) 4.99, (6) 5.32, (7) 5.25, (8) 5.03, (9) 5.09, (10) 5.11,

(11) 4.59, (12) 4.78, (13) 5.03, (14) 5.02, (15) 5.02, (16) 4.34, (17) 4.86, (18) 4.45, (X) 4.80

Positive additive effects indicate the Berk allele increased the trait, negative that the Berk allele decreased it.

% QTL variance = genetic variance at the QTL as a percent of the residual variance.


QTL results for meat quality traits on chromosome 15
F value
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Hormel Ham pH
Hormel Loin pH
Lab Loin pH
CM
SW1416
S0148
SW964
SW1683
SW936
SW1983
SW1119
EXHIBIT
A


QTL results for meat quality traits on chromosome 15
F value
0
1
2
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4
5
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7
8
1
5
9
13
17
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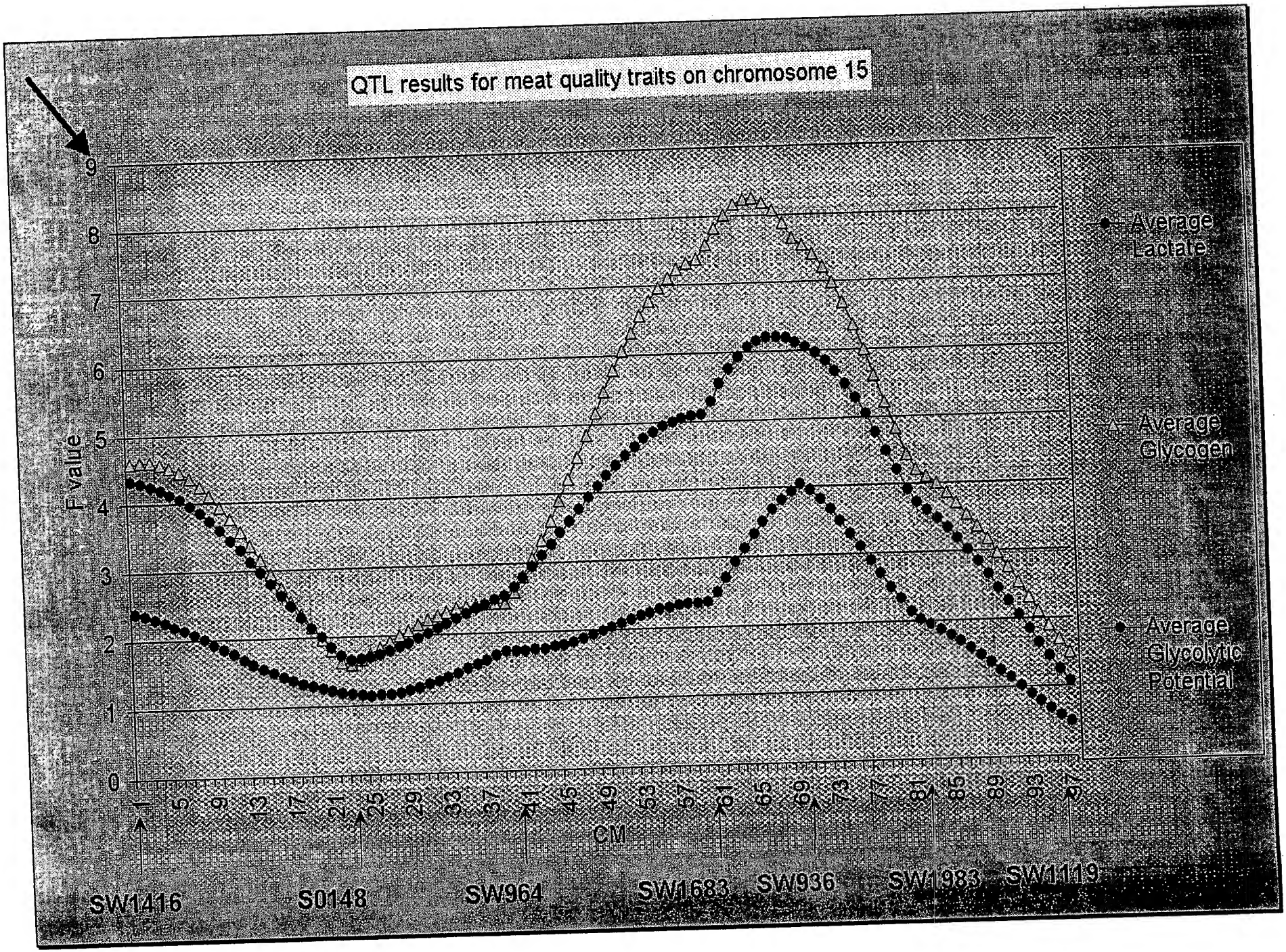
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97
CM
SW1416
S0148
SW964
SW1683
SW936
SW1983
SW1119
Average Star Probe Force
Tenderness Score
Flavor score


QTL results for meat quality traits on chromosome 15
F value
0
1
2
3
4
5
6
7
8
9
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5
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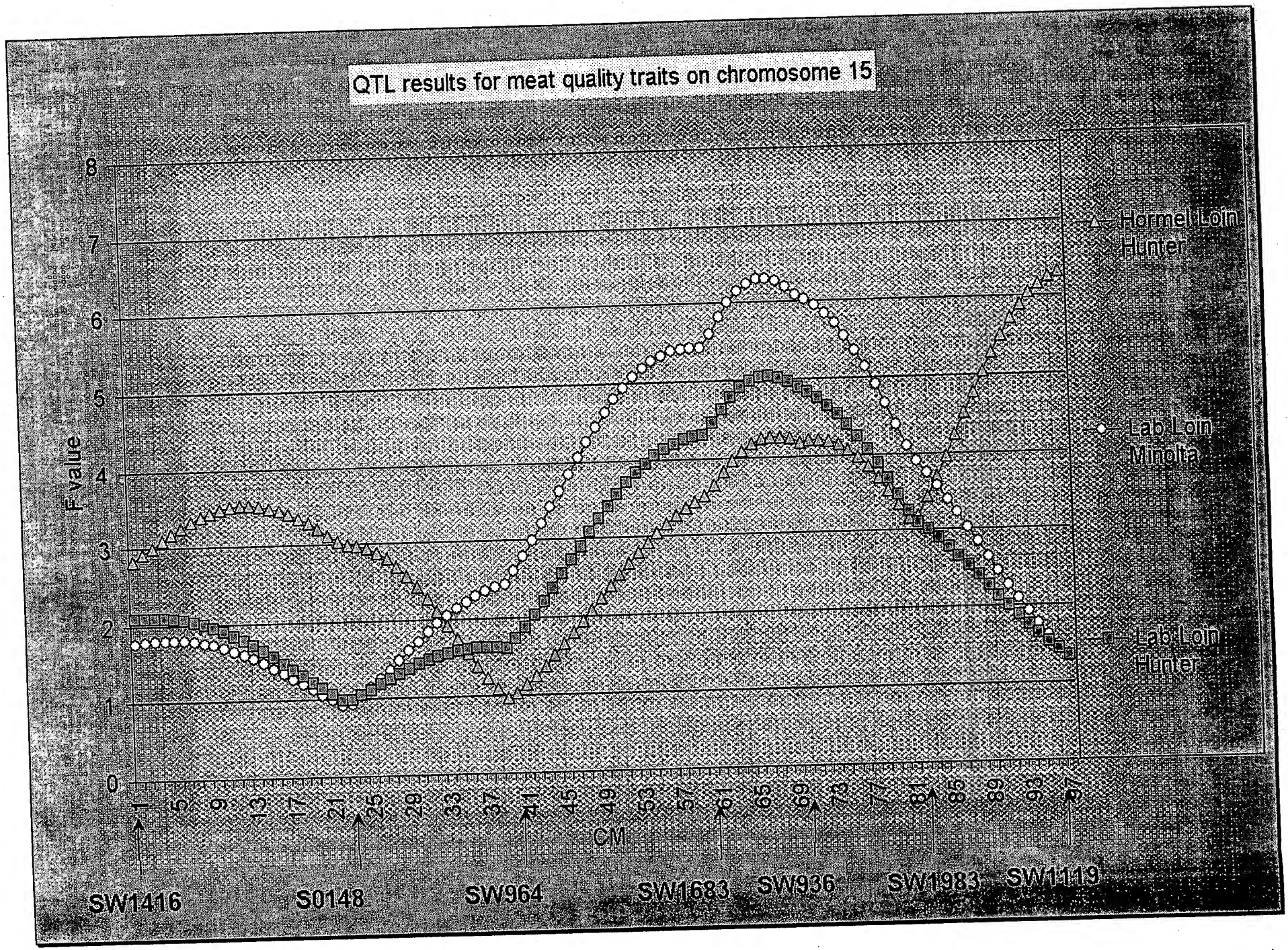
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97
CM
SW1416
S0148
SW964
SW1683
SW936
SW1983
SW1119
Average Lactate
Average Glycogen
Average Glycolytic Potential


QTL results for meat quality traits on chromosome 15
F value
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3
4
5
6
7
8
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97
CM
SW1416
S0148
SW964
SW1683
SW936
SW1983
SW1119
Hormel Loin Hunter
Lab Loin Minolta
Lab Loin Hunter

EXHIBIT E

RN project.

I used L. Andersson primers published in his paper (Sci

RNF 5' GGAGCAAATGTGCAGACAAG 3'

RNR 5' CCCACGAAGCTCTGCTTCTT 3'

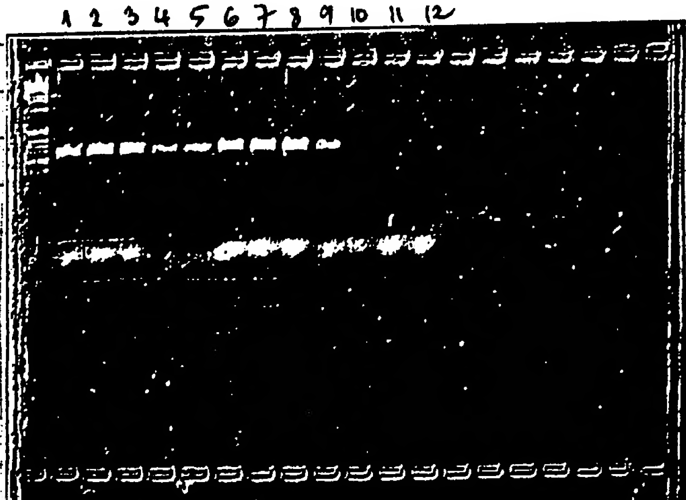
10x4=40
10x2=20
60

11x4=44
9x2=18
62

PCR	1x
PCR 10x buffer	1
Mg2+	1
dNTP's	1
RNF	0.25
RNR	0.25
Taq	0.07
Q	5.43

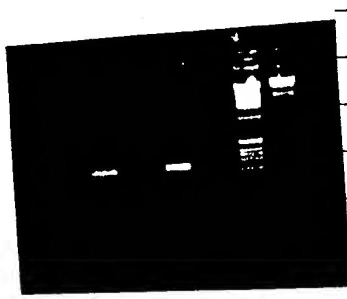
1. Cairina
2. Gold label
3. Count
4. Horizon
5. H 276
6. H 1821
7. H 3180
8. H 346
9. H 3705
10. -ve
11. } RT-PCR Thomas
12. } Boar

1 2 3 4 5 6 7 8 9 10 11 12



Bugs, Ann temp = 60°C.

I purified the product the MICROCON and after the conc. on gel I have the seq: No polymorphism codon 200 position. prods were cut.



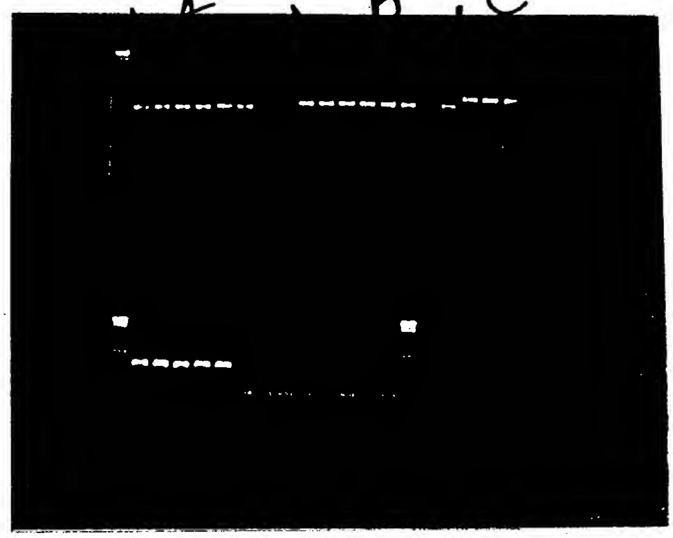
RN project:
New primers to explant Table RN52 and 398.

Combination:

	Size	Optimal Ann temp	
A: RF1 + R52R1	242	58.7	} RN5
B: RF1 + R52R2	270	58.4	
C: R391F + R391R	345	57.9	} RN3
D: R392F + R392R	134	56.9	

Machine: MJ-CKT → Program CH
: Ann temp (59)

A , B , C



PCR

	14x
PCR 10 x buffer	14
$MgCl_2$ (15mM)	14
dNTP's (2mM)	14
Primer Forward (A,B,C,D)	3,5
Reverse (A,B,C,D)	3,5
Taq	0,98
Q	78,02
	9,00 +
	1 µl DNA

A+B

1. ~~Cairina~~	3188	11
2. ~~Caunt~~	4491	12
3.	3167	12
4.	3247	12
5.	3106	11
6.	3102	11
7.	4908	22
8.	3381	12
9.	2571	11
10.	Caunt	22
11.	—	—
12.	Cairina	22
~~13.~~		
~~14.~~		

C+D

14 x 426 (7 animals in pair)

Set 25 → Plate II → Clon 5

Sample 1 – 8 → 1 – 8 Reaction

0,5µl from 1 – 8 9 – 12

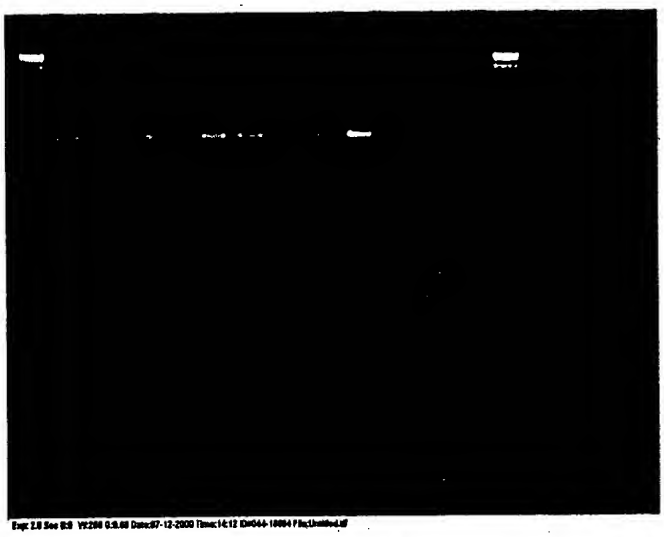
Digestion of RN-HphI number 52.

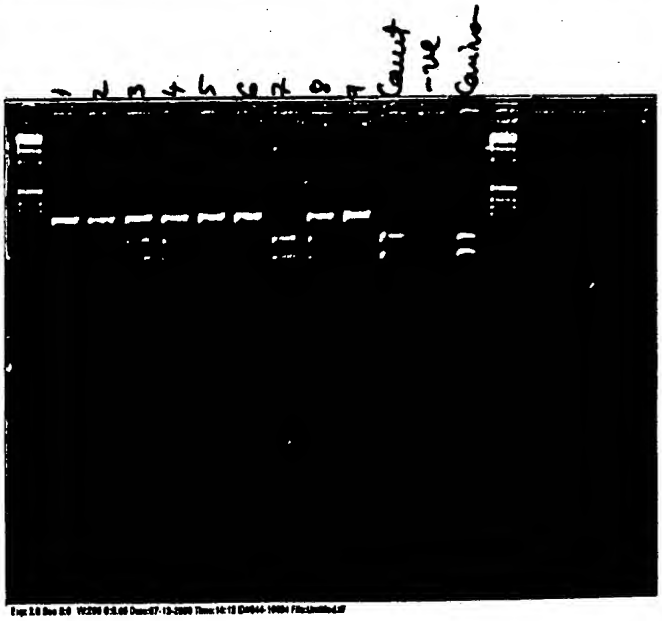
Primers used: B → RF1 + R52R2. (270 bp)

Digestion	1 X	12 X
Buffer NEB4	1	12
HphI	0.6	7.2
dH2O	5.4	64.8

+ 3 µl PCR product

Incubate: 37°C → 3 hr.

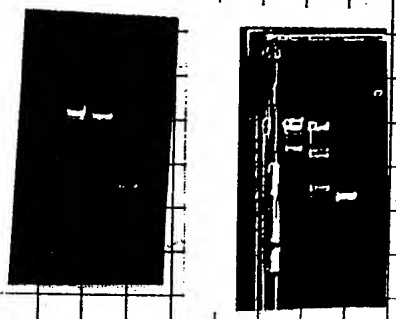




RW-398 marker purification

→ MICROCON filter.

→ 1 µl PCR product C (pag 143) diluted with primers: R391F
R391R



Conc. 30 ng/µl.

B X Y F2 animals tested for RN - HpaII Marker

Primers: RF1 + R52R2

	1X	48
PCR 10x buffer	1	48
MgCl	1	48
dNTPs	1	48
RF1	0,25	12
R52R2	0,25	12
Taq	0,07	3,36
H2O	5,43	260,64
		432/8 = 54

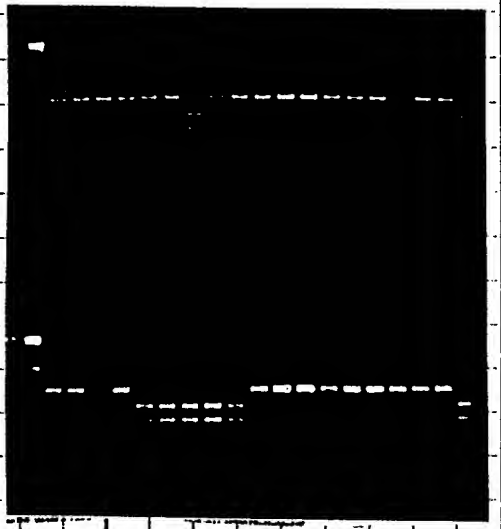
PCR check.

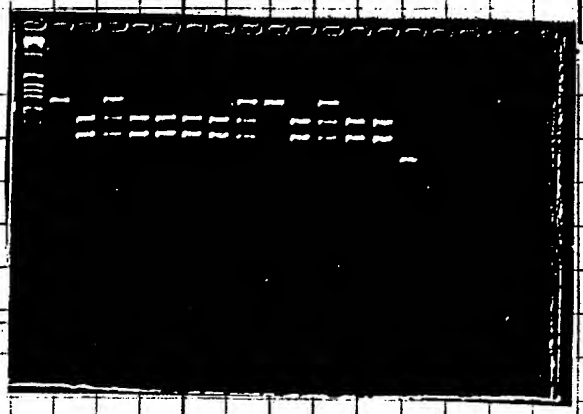
GL. Hon's -ne

Digestion

	1X	60X
Buffer NEB4	1	60
HpaII	0,6	36
H2O	5,4	324
		420 + / 52,5

3µl PCR product





	1	2	3	4	5	6	7
A	2780-3 12	1730-3 12	5580-3 12	526-2 24	432-3 11	438-2 12	604-3 12
B	1486-2 12	2046-2 12	1491-3 12	524-2 24	980-3 11	2232-2 22	5884-2 22
C	4623-2 12	2455-2 11	483-3 22	5233-2 24	3882-2 12	457-2 22	Plasmid 22
D	1405-2 12	2025-3 11	5803-3 12	6945-2 22	1523-3 12	3447-3 22	-ve
E	391-2 12	2024-2 12	1859-2 12	740-3 12	1158-3 11	2273-3 22	
F	2480-3 12	2453-3 12	1826-3 22	6822-2 24	2182-2 11	2424-3 12	
G	3882-2 22	1980-3 11	1889-3 11	1858-2 11	3552-2 11	2284-3 11	
H	4261-2 12	1859-2 22	1736-3 24	554-3 12	1923-3 24	4337-3 22	

Zufa: These animals were from F2 B x Y cop and are with TV lactate and glyc. potential.

148 | AN52 → Hauled on backside

4789	14.45	111.10	140.00	17892	Lact_best	11
9953	10.37	110.06	130.80	6822	Lact_best	11
4636	13.90	110.05	137.84	20253	Lact_best	11
9998	11.03	108.29	130.36	9603	Lact_best	11
9982	13.60	108.21	135.41	6812	Lact_best	11
4779	10.72	107.96	129.39	19203	Lact_best	11
9046	7.15	59.67	73.97		Lact_low	11
9839	3.67	45.64	52.96		Lact_low	11
9825	3.59	26.30	33.49		Lact_low	11
	9.83	87.48	107.13			Average

4895	11.66	119.60	142.91	17303	Lact_best	12
4750	12.70	116.93	142.32	18592	Lact_best	12
4708	14.76	115.02	144.55	390397	Lact_best	12
4804	11.43	111.05	133.91	20243	Lact_best	12
4685	9.67	110.65	129.97	4662	Lact_best	12
4707	7.01	110.33	124.34	4013	Lact_best	12
4879	7.69	61.77	77.15	20463	Lact_low	12
4710	20.85	61.26	102.95	436297	Lact_low	12
9869	5.67	60.79	72.14		Lact_low	12
4703	8.82	60.04	77.67	438297	Lact_low	12
9814	5.06	58.17	68.29		Lact_low	12
9833	4.69	56.49	65.86		Lact_low	12
9031	5.67	53.14	64.48		Lact_low	12
9882	7.51	51.54	66.55		Lact_low	12
9870	8.71	50.69	68.11		Lact_low	12
	9.46	79.83	98.74			Average

4817	13.96	113.97	141.90	18582	Lact_best	22
4637	12.95	113.62	139.53	1972	Lact_best	22
9862	12.21	112.16	136.57	221298	Lact_best	22
4839	11.93	109.63	133.49	17362	Lact_best	22
4786	13.50	108.18	135.18	18363	Lact_best	22
4652	5.36	63.07	73.78		Lact_low	22
4732	11.14	61.81	84.07	5233	Lact_low	22
9995	4.68	61.65	71.01		Lact_low	22
4897	7.72	61.45	76.88	27323	Lact_low	22
4663	4.30	61.36	69.94		Lact_low	22
4737	6.77	56.07	69.59		Lact_low	22
9832	2.01	40.55	44.57		Lact_low	22
	8.88	80.29	98.04			Average

150

RN-project

		x56
PCR 10x buffer		56
15mM $MgCl_2$		56
dNTP's		56
RNF		14
RNR		14
Taq		3,92
Q		304,08

504/8 = 63.

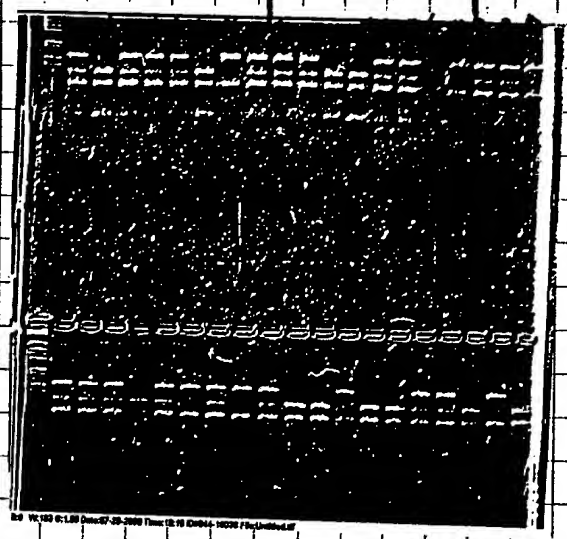
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

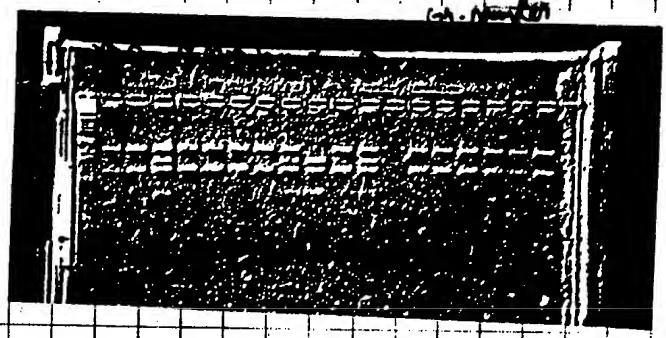
Digestion with BsaHI to differentiate the indian vha patient mutation in codon 199 of RN gene.

	1X	x56
Buffer NEB 4	1	56
BsaHI	0,3	22,4
BSA	0,1	5,6
H_2O	5,6	308,0
	7 µl PCR	392

Digit band under ear like gel : 1 - 23 25 - 39 Thirst

: 24 and 40 - end Second





4 RN100 – ranked all glyc. pot.

4817	13.96	113.97	141.90		Gly_best	6	11
4637	12.95	113.62	139.53		Gly_best	10	11
9862	12.21	112.16	136.57		Gly_best	12	11
4839	11.93	109.63	133.49		Gly_best	18	11
4652	5.36	63.07	73.78	3143	Gly_low	4	11
4778	4.37	63.13	71.87	19273	Gly_low	7	11
9995	4.68	61.65	71.01	9633	Gly_low	9	11
4663	4.30	61.36	69.94	398297	Gly_low	10	11
4737	6.77	56.07	69.59	5243	Gly_low	11	11
9832	2.01	40.55	44.57	3833	Gly_low	19	11
	7.85	**79.52**	**95.22**				
4841	22.73	100.68	146.14	24243	Gly_best	1	12
4895	11.66	119.60	142.91		Gly_best	4	12
4750	12.70	116.93	142.32		Gly_best	5	12
4705	16.30	107.72	140.32	391297	Gly_best	8	12
4786	13.50	108.18	135.18		Gly_best	14	12
4683	17.53	99.55	134.61	4623	Gly_best	15	12
4804	11.43	111.05	133.91		Gly_best	16	12
4702	13.61	106.56	133.79	4052	Gly_best	17	12
9901	1.04	72.37	74.45	7103	Gly_low	1	12
4731	5.00	64.33	74.32	5262	Gly_low	2	12
4806	4.55	64.23	73.34	21433	Gly_low	5	12
9869	5.67	60.79	72.14	5503	Gly_low	6	12
9871	1.93	67.24	71.08	4913	Gly_low	8	12
9814	5.06	58.17	68.29	2333	Gly_low	12	12
9870	8.71	50.69	68.11	5513	Gly_low	13	12
9882	7.51	51.54	66.55	438298	Gly_low	15	12
9833	4.69	56.49	65.86	3803	Gly_low	16	12
9031	5.67	53.14	64.48	27013	Gly_low	17	12
	9.41	**81.63**	**100.43**				
4708	14.76	115.02	144.55		Gly_best	3	22
4850	16.78	107.72	141.27	24152	Gly_best	7	22
4789	14.45	111.10	140.00		Gly_best	9	22
4636	13.90	110.05	137.84		Gly_best	11	22
9982	13.60	108.21	135.41		Gly_best	13	22
9046	7.15	59.67	73.97	27943	Gly_low	3	22
9839	3.67	45.64	52.96	432398	Gly_low	18	22
9825	3.59	26.30	33.49	221398	Gly_low	20	22
	10.99	**85.46**	**107.43**				

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IPRT (Non- Ames Lab) Employees: Please complete this form and return to ISU's Office of Intellectual Property and Technology Transfer, 310 Lab of Mechanics; 294-4740; FAX 294-0778; email: disclose@iastate.edu

A. Inventor/Creator(s) (attach more pages if necessary): Please call OIPTT if you need help in determining inventorship. Intellectual contribution is the most essential criterion.

ISU Inventor/Creator(s): Please designate corresponding inventor with an asterisk (*) behind his/her name. The corresponding inventor should be able to answer questions on both the technology and its commercial utility.

1. Name: Max F. Rothschild
   Dept/Affiliation: Animal Science
   Campus Address: 2255 Kildee Hall
   Campus Phone: 294-6202
   Home Address: 511 Oliver Circle
   Ames, IA
   Social Security No. : 547-72-6958
   Citizenship: USA

   Social Security No.: _____
   Citizenship: _____

EXHIBIT
F

2. Name: Daniel C. Ciobanu
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   Campus Phone: 294-9085
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   , Ames, IA
   Social Security No. : 479-27-8894
   Citizenship: Canada

4. Name: _____
   Dept/Affiliation: _____
   Campus Address: _____
   Campus Phone: _____
   Home Address: _____

5. Name: _____
Dept/Affiliation: _____
Campus Address: _____
Campus Phone: _____
Home Address: _____
_____
Social Security No. : _____
Citizenship: _____

6. Name: _____
Dept/Affiliation: _____
Campus Address: _____
Campus Phone: _____
Home Address: _____
_____
Social Security No. : _____
Citizenship: _____

7. Name: _____
Dept/Affiliation: _____
Campus Address: _____
Campus Phone: _____
Home Address: _____
_____
Social Security No. : _____
Citizenship: _____

8. Name: _____
Dept/Affiliation: _____
Campus Address: _____
Campus Phone: _____
Home Address: _____
_____
Social Security No. : _____
Citizenship: _____

Non-ISU co-inventor/creator(s): Please describe briefly the nature of the intellectual contributions by these Non-ISUers. Please note that the simple provisions of funding, research materials, equipment, assistance in routine statistical analyses, assistance in routine testing, and standard protocols, and bringing to your attention the existence of a problem but not the solution DO NOT constitute intellectual contribution.

Provided us with some additional details and ideas on the presence of the beneficial allele

1. Name: Graham S. Plastow
Business Affiliation: PIC
Business Address: PIC International Group
Fyfield Wick, OX13 5NA, UK
Business Phone: _____
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_____
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Citizenship: _____

3. Name: _____
Business Affiliation: _____
Business Address: _____
_____
Business Phone: _____
Home Address: _____
_____
Social Security No. : _____
Citizenship: _____

4. Name: _____
Business Affiliation: _____
Business Address: _____
_____
Business Phone: _____
Home Address: _____
_____
Social Security No. : _____
Citizenship: _____

Iowa State University Intellectual Property Disclosure & Record

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B. Title of Invention/Creation:

Associations between alleles *PRKAG3-199* and *PRKAG3-52* with positive meat quality in pigs.

Is this related to any previously disclosed invention/creation? If yes, please provide additional information.

Yes previous meat quality genes, see earlier disclosure. Also probably the authors Milan et al. (2000, Science, 288, 1248-1251) have applied for patenting the results of their research.

C. Reduction to Practice : Have you shown that the invention actually works as intended ? (i.e. have test results to demonstrate, have built a working prototypes, etc.)

X **Yes, already done** If yes, give date first successfully done: 13 and 19 July 2000 ;
Written record in: Lab notebook 122,143-148,150-151 and 4 (Notebook No. & page, file, n etc.)

☐ **No** (but working on it.)
☐ **No** (just an idea, nothing has been done.)

D. Brief Description of Invention/Creation (i.e. What is it ? What does it do ? What is it for ? Why was it invented/created ? etc.)

We have developed a test for two genetic markers (*PRKAG3-199* and *PRKAG3-52*) with expected influence on pork quality traits.
The tests include amplification by PCR of two fragments of the *PRKAG3* gene from porcine DNA and digestion with *HphI* (for *PRKAG3-52*) and *BsaHI* (*PRKAG3-199*) to determine different alleles. Then we determined the association of the alleles with different phenotypes. One allele in each locus/marker is associated with preferred traits.

Suggest some keywords : PCR-RFLP, selectable marker for economic traits, *PRKAG3*, Swine, pork quality
(to assist us in computer search)

If we are to file a patent application, we must have your data proving that the new invention actually works ('enabling data'), and also a detailed description of the materials and methods you used to collect the data. Please send us a copy of the 'enabling data' and your 'materials and methods.' If you have a manuscript ready for submission describing the invention/creation, please send us a copy.

See attached.

If your invention needs/consists of one or more original **software programs** or **computerized databases**, please put in the program/database a 'first screen' stating the following:

[Name of Program/Database]
by
[Name(s) of Creator(s)]

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E. What do you see as the mostly likely COMMERCIAL use(s) of your invention/creation ? (Update us when you think of new uses.)

As a genetic marker for marker assisted selection programs in pigs to improve meat quality.

F. Prior Art : (To determine whether we can protect your invention/creation, it will be necessary to compare it to what is already known or available. Please provide the following information to the best of your ability.)

i. What is the deficiency in the prior art which your invention/creation improves upon, or the limitation it extends ? (i.e. It works faster; is cheaper to make; produces less toxic wastes, etc.)

Prior art is only for other genes/markers controlling meat quality in pigs. In addition there is pr art for this gene but we have discovered a new variant to be used in other breeds.

ii. If you can, please provide us copies or references to the prior art (including patents, journal articles, book chapters, news releases, meeting abstracts, names of persons, etc.)

See references.

G. What are some other COMPETING invention/creation(s) & how do they compare to yours ?

In the same gene (PRKAG3) Milan et al (2000, Science, 288, 1248-1251), found a mutation in the 200 codon position affecting glycolitic potential and several meat quality traits in Hampshire pigs. Our mutation applies to many other breeds – not just Hampshire

H. What firms or types of companies do you think may be interested in your invention/creation?

PIC

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I. Conception: date you first got the idea: _____

J. Date & Form of First Written Record: _____
(date you first wrote down the idea or tried to make it work, what you wrote on--e.g. notebook no. & page, file, report, etc.)

Was the written record witnessed ? (check one) Yes X No ☐

K. First Public Disclosure : Have you told/written or are you planning to tell/write anybody about the invention/creation? (e.g. abstracts, presentations, proceedings, publications, etc.)
Yes No X - some details pointing to this have been released but not that a specific gene/allele was involved.

If yes, details of the EARLIEST incidence:

Date: _____ Event: _____
(name of event, place, sponsor etc.)

If possible, please provide a copy of the material you presented or will be presenting.

L. Funding Information: Since by contracts with the University most sponsors have certain intellectual property rights and require notification when an invention or a creation is made, it is important that ALL sources of funding utilized in the conception, creation, or enabling the invention/ creation be reported to this office. You are therefore **REQUIRED to disclose ALL relevant funding** sources below:

For **federal** funding agencies, give Agency Contract No. _____

For **all** funding sources, give ISU Grant No. _____

☐ DOE ☐ USDA ☐ NSF ☐ NIH ☐ EPA ☐ NASA ☐ DOC

Other (give full name): _____

IAHEES Projects: Project No.3609 _____ ; Source: x[Hatch ☐ McIntire-Stennis

IPRT Projects: Ames Lab/DOE Contract No. _____ CATD Project No. _____
Ames Lab B&R Code _____

Commodity Groups or Research Consortia : (i.e. USB, NPPC, EPRI, GRI, etc.)

Name of Group: _____

Industry (give company name) : PIC _____

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Has a research agreement been signed with this company ? XYes ☐No ☐Not Sure
If yes, a copy of the agreement should be sent to this office.- You have it.

If **NO external funding** (except for internal/University grants) received for the project, please check here: ☐

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M. Signatures/Assignment : All ISU inventor/creator(s) must sign.

Signature: [signature]	Date: 8/3/00	Signature: _____	Date: _____
Signature: [signature]	Date: 8/3/00	Signature: _____	Date: _____
Signature: [signature]	Date: 8/3/00	Signature: _____	Date: _____
Signature: [signature]	Date: _____	Signature: _____	Date: _____

By the signature(s) above and in accordance to the University policies, the party(ies) *hereby assigns to ISURF all intellectual property rights, titles, and interests* in this invention/creation.

N. Signatures of Non-ISU Inventor/Creator(s) :(Ames Laboratory funded inventions only)

Signature: [signature]	Date: 8/3/00	Signature: _____	Date: _____
Signature: _____	Date: _____	Signature: _____	Date: _____

O. Witnesses : (at least one person not directly involved in the invention/creation)

Signature:	Ann M Shuey	Signature:	_____
Printed Name:	Ann M Shuey	Printed Name:	_____
Date:	8/3/00	Date	_____

For Ames Laboratory Office of Intellectual Property Use Only:

Forwarded by: _____ **Date:** _____

APPENDIX I (CONFIDENTIAL)

Introduction

AMP-activated protein kinase is involved in turning on ATP-producing pathways and inhibits ATP-consuming pathways. Also it can inactivate glycogen synthesis by phosphorylation. AMPK is composed of three subunits: the catalytic α chain and two regulatory subunits β and γ.

Recently the sequence of a gene encoding an isoform of the regulatory γ subunit of pig AMPK (*PRKAG3*) was published (Milan et al., 2000). The identification of the gene allowed researchers to genetically map the gene to pig chromosome 15 using DNA markers. This provided the basis for linked marker tests where the presence of the acid meat gene (RN^-) could be tracked.

Milan et al. (2000) found a mutation (in codon 200) in the *PRKAG3* gene associated (in homozygous status) with high glycolytic potential in Hampshire pigs or RN^- phenotype. The pigs with this phenotype have a low ultimate pH, a reduced water holding capacity and give a reduced yield of cured cooked ham. These effects are due to a ~70% increase in muscle glycogen content in RN^- animals (genotype RN^-/RN^-). In some forms of ham processing there up to 5% decreasing of the yield due to the mutation (or acid meat gene) presence in homozygous status. Breeders could now use these tools to manipulate the frequency of the allele in their breeding herds and in slaughter pigs.

However, this test is specific for this "defective" gene and analysis of different lines of pigs suggests that this mutation arose in the Hampshire breed and is in very low frequency or non existant in other breeds. This is almost certainly the result of introgression (crossing in) of the gene from the Hampshire breed.

Very recently Malek et al. (2000) reported interesting QTLs for lactate, glycogen and glycolytic potential on chromosome 15, based on a Berkshire x Yorkshire 3 generation family experiment. These QTLs were mapped exactly on the *PRKAG3* gene position. Based on the fact that Berkshire and Yorkshire are considered free of RN^- phenotype, we assumed the presence of a third allelic variant of the *PRKAG3* gene affecting lactate, glycogen and glycolytic potential and due to these high meat quality traits in pigs.

APPENDIX II (CONFIDENTIAL)

PRKAG3-52 PCR-RFLP Test

HphI polymorphism

Primers

RF1 - 5'ATG AGC TTC CTA GAG CAA GGA G 3'
RN52R2 - 5'GGC TGC ATG ATG TTA TGT GCC T 3'

PCR conditions

Mix1

10x PCR buffer	1.0 µl
$MgCl_2$ (15mM)	1.0 µl
dNTPs (2mM)	1.0 µl
RF1 primer (10pm/µl)	0.25 µl
RN52R2 primer (10pM/µl)	0.25 µl
Taq polymerase (5U/µl)	0.07µl
ddH_20	5.43 µl
genomic DNA	1 µl

Combine the Mix1 and DNA in a reaction tube. Overlay with mineral oil. Run the following PCR program: 94°C for 4 min.; 35 cycles of 94 °C for 45 sec., 59 °C for 45 sec and 72 °C for 45 sec; followed by a final extension at 72 °C for 12 min.
Check 3 µl of the PCR on a 2% agarose gel to confirm amplification success and the clean of the negative control. Product size is 270bp.
Digestion can be performed by the following procedure:

***Hph*I digestion reaction**

PCR product	3 µl
NE Buffer 4	1 µl
HphI(5U/µl)	0.6 µl
ddH_20	5.4 µl

Make a coktail of PCR product, buffer, enzyme and water. Incubate for 2 hours at 37 °C.
Mix the digested product with loading dye (1:6) and run on a 4% agarose gel.

Genotypes:

11 – 270bp
12 –270bp, 158bp and 112bp
22 – 158bp and 112bp.

APPENDIX III (CONFIDENTIAL)

PRKAG3-199 PCR-RFLP Test

BsaHI polymorphism

Primers

RNF - 5' GGA GCA AAT GTG CAG ACA AG 3'
RNR - 5' CCC ACG AAG CTC TGC TTC TT 3'

PCR conditions

Mix1

10x PCR buffer	1.0 µl
$MgCl_2$ (15mM)	1.0 µl
dNTPs (2mM)	1.0 µl
RNF primer (10pm/µl)	0.25 µl
RNR primer (10pM/µl)	0.25 µl
Taq polymerase (5U/µl)	0.07µl
ddH_20	5.43 µl
genomic DNA	1 µl

Combine the Mix1 and DNA in a reaction tube. Overlay with mineral oil. Run the following PCR program: 94°C for 4 min.; 35 cycles of 94 °C for 45 sec., 61 °C for 45 sec and 72 °C for 1 min; followed by a final extension at 72 °C for 12 min.
Check 3 µl of the PCR on a 2% agarose gel to confirm amplification success and the clean of the negative control. Product size is 258bp.
Digestion can be performed by the following procedure:

***BsHI* digestion reaction**

PCR product	3 µl
NE Buffer 4	1 µl
BsaHI(5U/µl)	0.6 µl
BSA (10mg/ml)	0.1 µl
ddH_20	5.3 µl

Make a coktail of PCR product, buffer, enzyme and water. Incubate for 2 hours at 37 °C.
Mix the digested product with loading dye (1:6) and run on a 4% agarose gel.

Genotypes:

11 – 167bp and 91bp.
12 –167bp, 119bp and 91bp
22 – 119bp and 91bp.

APENDIX IV (CONFIDENTIAL)

Results

Phases:

I. We sequenced the entire *PRKAG3* gene using RT-PCR and analyzing samples from the Berkshire x York 3 generation family (see suporting QTL graph and table which outlines region of gene – Malek et al.,2000) but also samples from Duroc and Meishan pig breeds. We did not find in all the analyzed samples, the presence of the "acid meat" mutation in the codon 200 of the *PRKAG3* gene. In order to find the causative mutation of the phenotypic variation of lactate, glycogen and glycolytic potential, we looked for a possible mutation in the coding region involved the observed phenotype. We found a new mutation in the 154 position (codon **52** – *PRKAG-52*) of the gene where the nucleotide carrying a guanine is changed to adenine. This mutation changes an amino acid in the peptide sequence: glycine changed to serine. Also we considered as a possible causative mutation the one discovered by Milan et al (2000) in 1845 position of the gene (codon **199** – *PRKAG-199*). This mutation also changes an amino acid: valine to isoleucine.

II. Using a PCR-RFLP test, for each mutation we tested F_2 samples with extreme phenotypes for lactate (the highest QTL lod), from Berkshire x Yorkshire 3 generation family.

	n	Glycogen	Lactate	Glycolytic potential
PRKAG3-52				
11	9	9.83	87.48	107.13
12	15	9.46	79.83	98.74
22	12	8.88	80.29	98.04
PRKAG3-199				
11	10	8.19	79.35	95.72
12	15	9.47	76.12	95.06
22	10	10.22	90.50	110.93

The results suggested that in the case of *PRKAG3-52* locus the allele 2 could be associated with lower glycogen, lactate and glycolytic potential. In the case of *PRKAG3-199* locus the allele *1* could be associated with lower glycogen, lactate and glycolytic potential. These alleles we consider to be "hypoglycolitic" and could be associated with high meat quality traits. The animals with this "third/fourth" allele have the potential to produce meat of the highest technical quality in terms of color, pH, and drip loss etc. These polymorphisms (at amino acid 52 and amino acid 200 or is it base) can be used alone or in combination. Most importantly, these polymorphisms are segregating in the breeds commonly used for commercial pig meat production. Thus this present invention is more widely applicable than the RN⁻ test, which is effectively limited to the Hampshire breed. The association of these alleles with better meat quality, allows utility in further improving the quality of pork products by utilizing marker assisted selection programs.

Table. Evidence for QTL for various growth and meat quality traits by chromosome. Estimated significance level (F value) for trait QTL.

SSC	Trait	F-value	Location	Additive effect	S.E.	Dominance effect	S.E.	% QTL var
15	Hormel Loin Hunter	6.31	96	-1.065	0.321	0.624	0.500	3.16
15	Lab Loin Hunter	5.04	66	-0.677	0.216	0.166	0.327	2.46
15	Lab Loin Minolta	6.30	66	-0.727	0.207	0.165	0.313	3.05
15	Hormel Ham pH	8.42	72	0.054	0.014	-0.021	0.021	4.00
15	Hormel Loin pH	12.15	76	0.053	0.011	-0.005	0.015	5.61
15	Lab Loin pH	9.05	45	0.043	0.012	-0.038	0.019	5.14
15	Average Glycogen (*u*mol/g)	8.25	65	-0.771	0.222	0.708	0.337	4.27
15	Average Glycolytic Potential (*u*mol/g)	6.21	67	-3.666	1.048	0.766	1.587	2.95
15	Tenderness Score	5.22	44	0.240	0.084	-0.204	0.135	3.00
15	Average Star Probe Force (kg)	5.25	42	-0.166	0.054	0.092	0.085	2.88
15	Flavor score	6.41	91	0.355	0.114	-0.336	0.183	3.73

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[1] Chromosome-wise F-statistic thresholds at the 5% level, as determined by permutation test were as follow:
(1) 5.08, (2) 5.12, (3) 5.14, (4) 5.14, (5) 4.99, (6) 5.32, (7) 5.25, (8) 5.03, (9) 5.09, (10) 5.11, (11) 4.59, (12) 4.78, (13) 5.03, (14) 5.02, (15) 5.02, (16) 4.34, (17) 4.86, (18) 4.45, (X) 4.80
Positive additive effects indicate the Berkshire allele increased the trait, negative that the Berkshire allele decreased it.
% QTL variance = genetic variance at the QTL as a percent of the residual variance.